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The Cellular Interactions of PEGylated Gold Nanoparticles: Effect of PEGylation on Cellular Uptake and Cytotoxicity

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The Effect of PEGylation on Cellular Uptake and Cytotoxicity of Gold Nanoparticles.

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Supporting Information.

I) Synthesis, purification and characterization of PEGylated Au NPs.

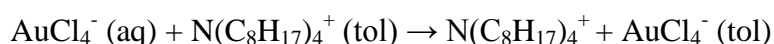
II) Cell-nanoparticle interactions studies

III) References

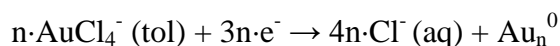
I) Synthesis, purification and characterization of PEGylated Au NPs.

I.1) Synthesis of hydrophobic gold nanoparticles (Au NPs).

Gold nanoparticles (Au NPs) were synthesized according to some modifications of the two-phase Brust method as described in previous publications^[1]. All glassware was cleaned with aqua regia, carefully rinsed with MilliQ water and dried before use. The reaction was carried out at room temperature under ambient conditions. Hydrogen tetrachloroaurate (III) (0.300 g, 0.88 mmol 99.9%, Alfa Aesar #12325) was dissolved in 25 mL of Milli-Q water, obtaining the aqueous solution of gold precursor. Then, a solution of tetraoctylammonium bromide (TOAB, 2.170 g, 3.9 mmol, Sigma-Aldrich #29,413-6) in 80 mL of toluene (Fluka #89682) was prepared. Both solutions of hydrogen tetrachloroaurate and TOAB were mixed in a 250 mL separation funnel, and shaken vigorously for about 5 min. The AuCl_4^- ions were gradually transferred into the organic phase (tol), due to the formation of tetraoctylammonium- AuCl_4^- ion pairs. In this process, the initial colourless toluene phase turned into a deep orange colour while the initial yellow coloured aqueous phase (aq) turned colourless.

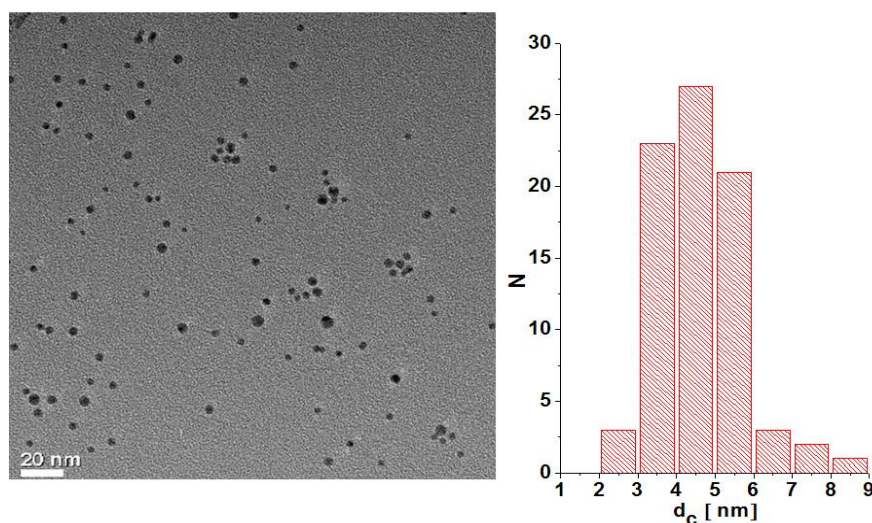


The aqueous solution was discarded and the toluene solution was transferred to a 250 mL round flask. A freshly prepared solution of sodium borohydride (0.334 g, 0.88 mmol, Sigma #452882) in 25 mL of Milli-Q water was added dropwise within one minute to the vigorously stirred solution of gold precursor in toluene. A few seconds after the addition, the color changed from deep orange to red-violet, indicating the nucleation of gold clusters mediated by reduction of the gold ions by sodium borohydride.



The solution was kept stirring for one hour and then was transferred to a clean 250 mL separation funnel where the aqueous phase was discarded. Then, 25 mL of 0.01 M HCl were added and the mixture was vigorously shaken to remove the excess of sodium borohydride. In a second step, 25 mL of 0.01 M NaOH were added in order to remove the excess of acid. Finally, 25 mL of Milli-Q water were added and shaken to remove the ion excess. This step was repeated four times. The aqueous phase was discarded and the solution was transferred to a 250 mL round bottom flask. The solution was stirred overnight to get thermodynamically stable NPs with narrow size distribution, in a process called *Ostwald ripening*. After this, in order to enhance the NPs stability an additional ligand (surfactant) exchange step was carried out. For this purpose 10 mL (8.450 g, 41.7 mmol) of 1-dodecanethiol (Sigma #471364) was added and the mixture was then heated at 65 °C for 3 hours under stirring. Due to the high binding affinity of thiols to gold, dodecanethiol displaces the Br^- ions of the NP surface, yielding dodecanethiol-coated Au NPs. The solution was cooled down to room temperature and the larger agglomerates were removed by centrifugation at 2500 rpm for 5 min. The supernatant was collected and larger agglomerates in the bottom of the vial were discarded. Then, methanol was added to the collected supernatant, until the solution turned cloudy. The solution was then centrifuged at 2000 rpm for 5 min. After discarding the supernatant, the precipitate containing the Au NPs was redissolved in toluene upon vigorous shaking. Again cold methanol was added to the NP solution until it turned cloudy and the mixture was centrifuged for 5 min at 2000 rpm. The supernatant was discarded and the precipitate containing the purified Au NPs was redissolved in chloroform. The average diameter of the

inorganic core of the Au NPs was determined by transmission electron microscopy (TEM) to be $d_c = 4.6 \pm 1.1$ nm (**Supporting Figure S1**).

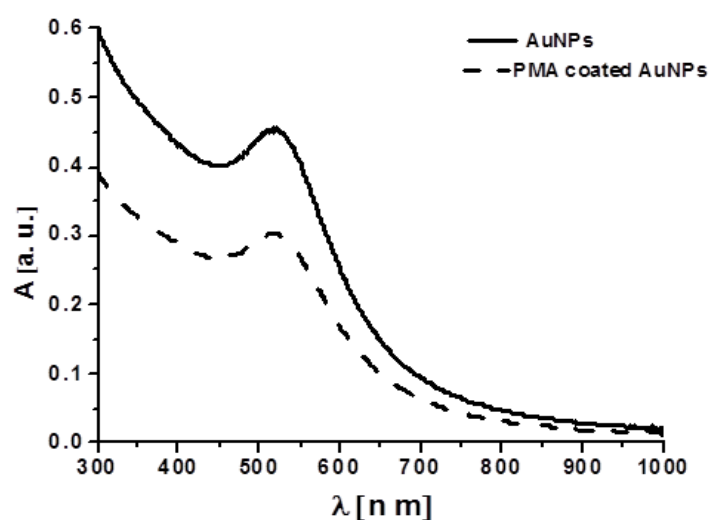


Supporting Figure S1: TEM image and size distribution of the Au NP cores with an average size of $d_c = 4.6 \pm 1.1$ nm. d_c denotes the diameter of the inorganic core (without surfactant) and N refers to the frequency of NP counts. The scale bar corresponds to 20 nm.

The Au NP concentration was determined by UV/vis absorption measurements (Agilent 8450 spectrometer) using the Beer-Lambert law:

$$A = \varepsilon \cdot l \cdot c$$

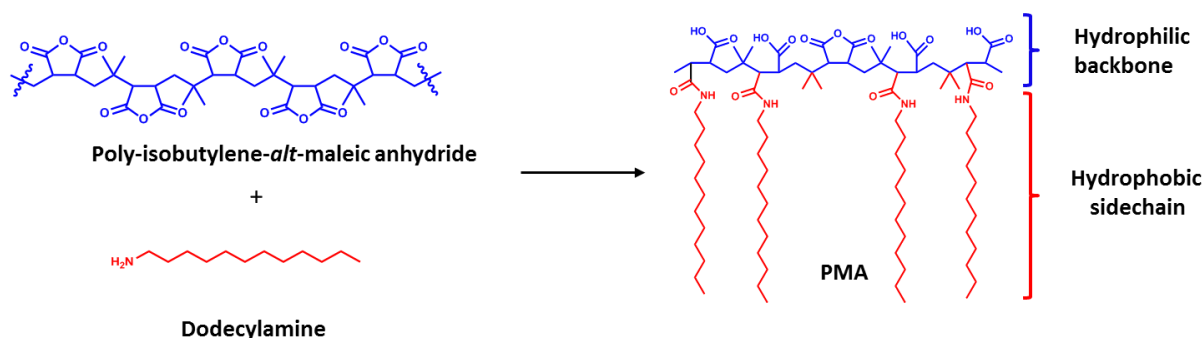
Here A is the absorbance, ε is the extinction coefficient of the sample [$M^{-1}cm^{-1}$], l is the path length [cm] and c the concentration of the sample [M]. For our sample, the extinction coefficient was assumed to be $\varepsilon = 8.7 \cdot 10^6 M^{-1}cm^{-1}$ at their plasmon peak at around 517 nm. The concentration c of the resulting Au NPs solution was in the micromolar range.



Supporting Figure S2: Absorption spectrum of the Au NPs before (solid line) and after the polymer coating (dashed line). The spectra are scaled for a better visualization (cf. §1.3).

I.2) Transfer of NPs to aqueous solutions. Synthesis of an amphiphilic polymer for polymer coating of NPs.

Testing the toxicity of Au NPs in biological samples requires its transfer to aqueous solutions. A commonly used strategy is the use of amphiphilic polymers^[2]. These amphiphilic polymers consists in a hydrophilic backbone where hydrophobic side chains are linked. The hydrophobic side chains of the polymer intercalates with the hydrophobic surfactant in the NPs surface, keeping the hydrophilic moiety pointing out the aqueous solution and providing solubility to the NPs. As the NPs stability is based on this hydrophobic interaction, this methodology can be applied to all kind of NPs cores synthesized with a hydrophobic surfactant^[3]. In this work, the polymer used is based on a hydrophilic backbone of poly(isobutylene-*alt*-maleic anhydride) which was functionalized with dodecylamine through formation of amide bonds upon reaction of the amines and the maleic anhydride groups (PMA polymer). This reaction leaves the surface functionalized with carboxylic acids and thus getting a negatively charged particle under basic conditions after polymer coating. A charged surface, positive or negative, leads to a highly stabilized colloidal NPs solution (See **Supporting Figure S3**).



Supporting Figure S3. Scheme of the PMA amphiphilic polymer synthesis.

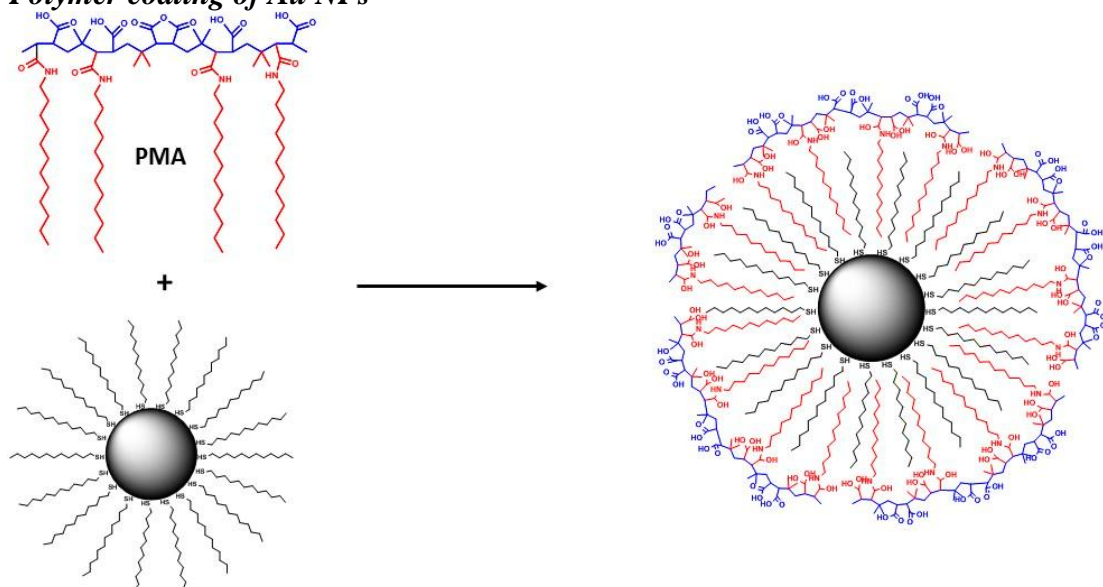
I.3) Synthesis of the amphiphilic polymer polyisobutylene-*alt*-maleic anhydride-dodecylamine (PMA) [25% anhydride - 75% NH_2C_{12}]

The polymer synthesis was carried out following our previously published protocols^[3b, 4]. The backbone of polyisobutylene-*alt*-maleic anhydride rings is covalently attached to dodecylamine *via* amide bonds (**Figure S3**). The polymer defined as [25% anhydride - 75% NH_2C_{12}] is designed in a way where the initial poly-isobutylene-*alt*-maleic anhydride chain is reacted with dodecylamine to get 75% of the initial maleic anhydride rings functionalized, leaving 25% of the groups intact. All the organic solvents used were anhydrous in order to keep the maleic anhydride rings active during the synthesis. Briefly, 2.70 g (15 mmol) of dodecylamine (98%, Sigma, # D22,220-8) were dissolved in 100 mL of anhydrous tetrahydrofuran (THF, $\geq 99.9\%$, Aldrich, #186562). This solution was poured into a 250 mL round bottom flask containing 3.084 g (20 mmol expressed as monomer) of poly(isobutylene-*alt*-maleic anhydride), (average M_w ~6,000 g/mol, Sigma, #531278). Each polymer chain contains an average of 39 anhydride (monomer) units.¹ The cloudy mixture was sonicated for

¹ As the molecular weight of one monomer unit of polyisobutylene-*alt*-maleic anhydride (maleic anhydride + isobutylene) is $MW = 154$ g/mol; $3.084\text{ g polymer} / 154\text{ g/mol (monomer)} = 0.02\text{ mol (monomer)} = 20\text{ mmol (monomer)}$. Each polymer molecule has around 39 monomer units / anhydride rings. The amphiphilic polymer

a few seconds (~20 s) and then heated at 60 °C for three hours upon stirring. This dodecylamine/anhydride rings ratio was chosen to get 75% functionalization, leaving 25% of the anhydride rings unreacted. Then, the solvent was concentrated in the rotary evaporator to a final volume of 30-40 mL and was heated at 60 °C overnight. Finally, the solvent was completely evaporated under reduced pressure and the resulting product was redissolved in 40 mL of anhydrous chloroform to a final (monomer) concentration of $c_P = 0.5$ M. In the synthesis, we assume that the reaction is quantitative. Considering a 75% modification with dodecylamine ($M_W = 185.36$ g/mol), the final molecular weight of the polymer is approximately 11400 g/mol.

I.3) Polymer coating of Au NPs



Supporting Figure S4: Schematic illustration of the coating of dodecanethiol-protected Au NPs with PMA amphiphilic polymer.

For the polymer coating of the Au NPs, Au NPs dispersed in chloroform were mixed with the amphiphilic polymer with conditions calculated as in previously published protocols^[3a]. As standard procedure, the amount of amphiphilic polymer (referred to in monomer units) was determined by the total surface area of Au NPs. Hereby the Au cores of the NPs were assumed as spheres with $d_c = 4.6$ nm diameter, and their concentration was obtained with the UV/vis absorbance analysis with $\epsilon = 8.7 \cdot 10^6$ M⁻¹cm⁻¹ (cf. §I.1). For the calculation of the overall surface area (A_0) of one NP it is important to consider the effective NP diameter (d_{eff}) as the sum of the metallic Au core and the surfactant. Using dodecanethiol as surfactant, we consider its length as $l_{surfactant} = 1.6$ nm, so the effective diameter was defined as:

$$d_{eff} = d_c + 2 l_{surfactant} \text{ (nm)}$$

In our case, $d_{eff} = 4.6 + 2 \cdot 1.6$ nm = 7.8 nm. The surface area of a single NP is:

$$A_{NP} = \pi \cdot d_{eff}^2$$

has a molecular weight of $M_w \sim 6000$ g/mol whereby the molecular weight of one polymer unit is $M_w \approx 154$ g/mol. Hence each amphiphilic polymer molecule comprises around $6000 / 154 = 39$ monomer units.

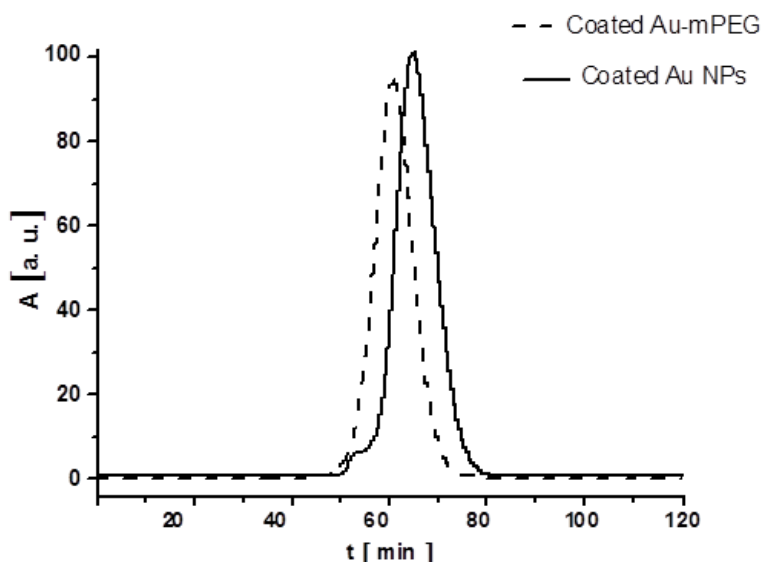
For our value of d_{eff} we obtain $A_{\text{NP}} = 191.13 \text{ nm}^2$. The total surface of the NP solution (A) is calculated using the formula:

$$A = c \cdot V \cdot N_A \cdot A_{\text{NP}}$$

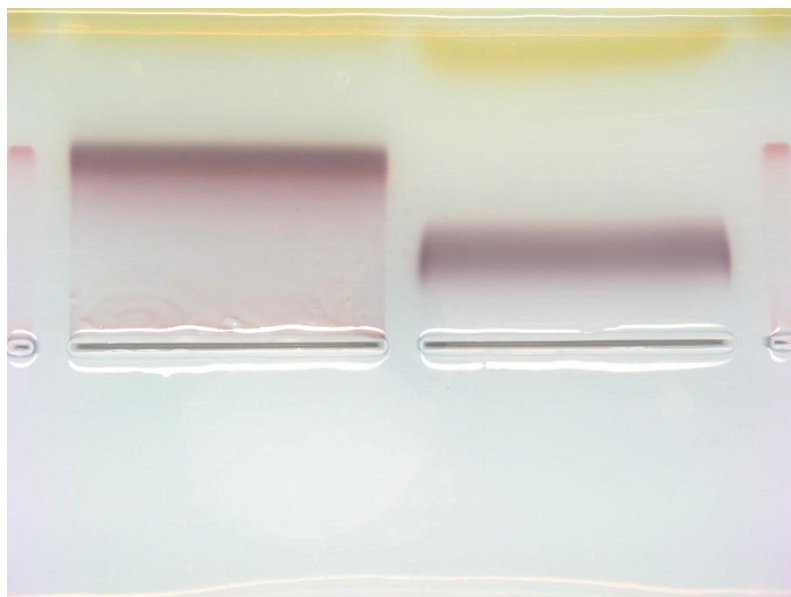
Where c is the concentration of colloidal NPs in the aliquot solution [mol/L], V is the volume of the aliquot [L], A_{NP} is the surface area of one single NP [nm^2] and N_A is the Avogadro constant, $6.02 \cdot 10^{23} \text{ mol}^{-1}$. For one 1 mL aliquot of $3.6 \text{ }\mu\text{M}$ solution of Au NPs, the area, calculated according to equation (3) is $4.14 \cdot 10^{17} \text{ nm}^2$. The amount of polymer was optimized to a ratio of polymer monomer units per surface area on NPs of $R_{\text{P/Area}} = 50 \text{ monomer units/nm}^2$. The volume of polymer solution $V_{\text{P}}[\text{L}]$ is then calculated according to the formula:

$$V_{\text{P}} = \frac{N_{\text{P}}}{c_{\text{P}}} = \frac{A \cdot R_{\text{P/Area}}}{N_A \cdot c_{\text{P}}}$$

Here N_{P} is the number of polymer monomers needed [mol], and c_{P} is the concentration of polymer monomers in the stock solution [M] which in the present case is 0.05 M. As standard procedure, to an aliquot of $V = 1 \text{ mL}$ and $c = 3.6 \text{ }\mu\text{M}$ of Au NPs in chloroform, $688 \text{ }\mu\text{L}$ of 0.05 M PMA polymer solution (V_{P}) was added. The mixture was manually stirred for five minutes and the solvent was completely evaporated under reduced pressure in a rotary evaporator. Then, 50 mM sodium borate buffer pH 12 (SBB 12) was added and the NPs sample was dissolved upon sonication. Basic pH and sonication induces the hydrolysis of the unreacted anhydride rings and thus enhance the NPs solubility. The NPs dispersion was concentrated using centrifuge filter tubes. The resulting water-soluble PMA-Au NPs were purified using size exclusion chromatography (SEC; **Supporting Figure S5**) and/or gel electrophoresis (**Supporting Figure S6**), as will be described in more detail below.



Supporting Figure S5: SEC elution profile of the polymer coated Au NPs before (solid line) and after modification with 2 kDa mPEG (dashed line) running through a size exclusion column. The absorption of the elute A is plotted versus the elution time t . Bigger size of mPEG coated Au NPs reduces the retention time of the particles in the column.



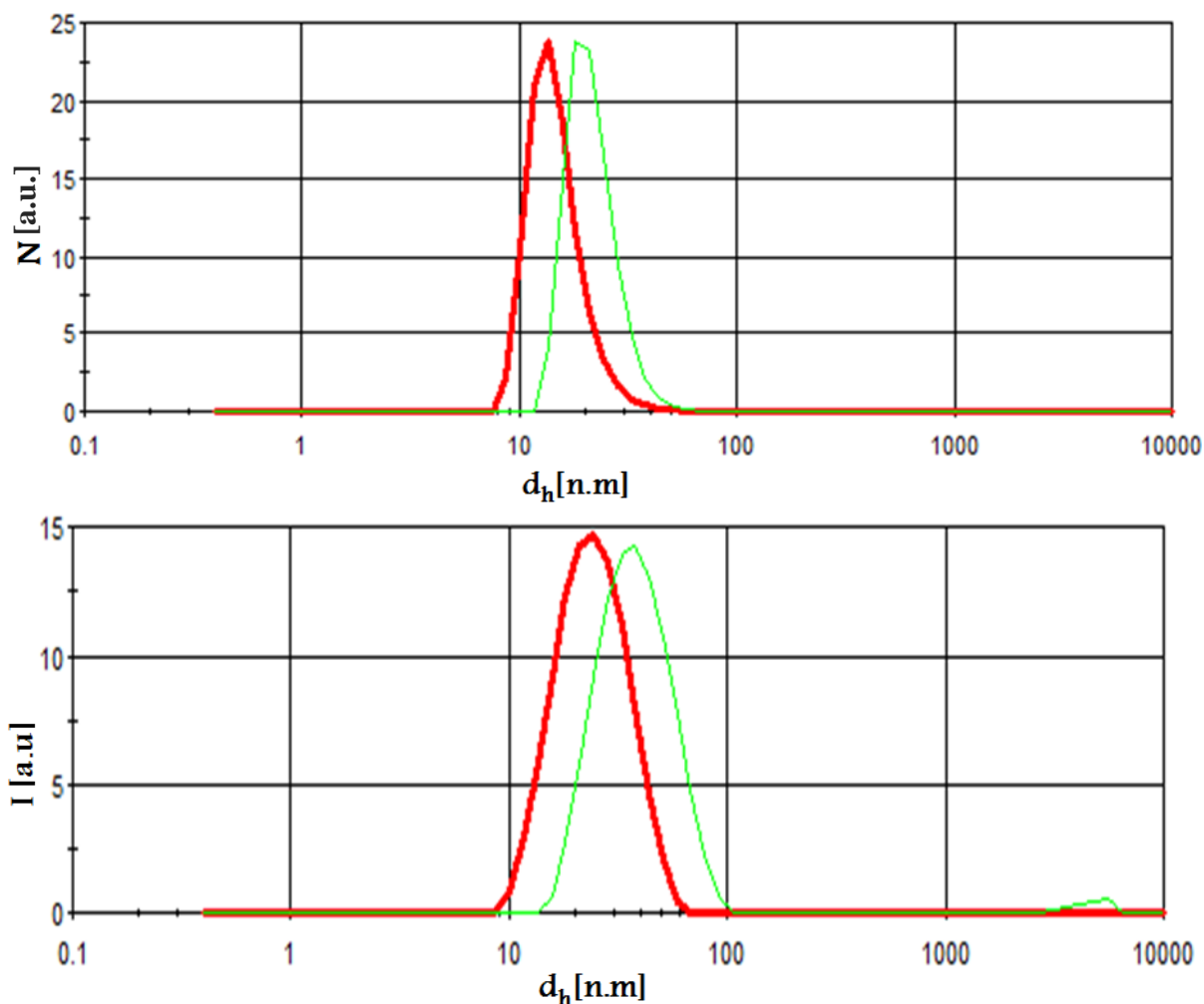
Supporting Figure S6: Gel electrophoresis image of the polymer coated Au NPs (left) and polymer coated Au NPs saturated with 2 kDa mPEG (right) on a 2 % agarose gel. The sample was run for 60 minutes at constant voltage 100 V (negative electrode on the bottom, positive electrode on top in the image). 10 nm phosphine coated Au NPs were run as control in the left and right lane. Au NPs functionalized with mPEG gain size and neat charge decrease, becoming retarded in the gel by comparison with the Au NPs not functionalized^[5].

1.4) Modification of the polymer coated NPs with 2kDa methoxy-polyethylene glycol (mPEG-NH₂; 2 kDa)

The polymer coated Au NPs were modified with mPEG-NH₂ via EDC chemistry in order to saturate the NPs surface using methodology previously published (see **Supporting Figure S7**)[11]. As standard procedure, in a vial, a 3.1 μ M solution of the polymer coated Au NPs in Sodium borate buffer (SBB) pH=9, (100 μ L, $3.1 \cdot 10^{-10}$ mol) was mixed with a freshly prepared solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Sigma Aldrich, # E7750) (397 μ L, 0.1 M). The mixture was shaken for 2 minutes and then a solution of mPEG-NH₂ -2 kDa (155 μ L, 1 mM; Rapp polymer, #12-2000-2) was added. The mixture was shaken and kept for 2 hours at room temperature. The molar ratio between the Au NPs : mPEG-NH₂ : EDC was calculated as at 1:500:128000. These molar ratios lead to mPEG saturated NPs, as established in previous publications^[6].

Sample	Hydrodynamic diameter d_h [nm]		Zeta potential [mV]
	Number distribution	Intensity distribution	
polymer-coated Au NPs	12.6 ± 1.1	14.1 ± 3.9	-31.9 ± 5.2
polymer-coated Au NPs modified with 2kD PEG-OCH ₃	21.7 ± 2.9	36.7 ± 6	-7.6 ± 0.8

Supporting Table S1: Hydrodynamic size d_h (as calculated from the number or intensity distribution) and the zeta potential of the polymer coated Au NPs before and after the modification with 2kD PEG-OCH₃ as measured in SBBpH9 using DLS.



Supporting Figure S8: Hydrodynamic diameter d_h of the polymer coated Au NPs before (red line) and after modification (green line) with mPEG of 2 kDa as determined with DLS. N refers to the number distribution $N(d_h)$ and I refers to the intensity distribution $I(d_h)$ measured in SBB9.

II) Cell-nanoparticle interactions studies

II.1) Cell culture.

C17.2 neural progenitor cells and PC12 cells were maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum, 5% horse serum, 2 mM L-Glutamine and 1% Penicillin/Streptomycin (Gibco, Invitrogen, Merelbeke, Belgium). Cells were maintained in a humidified atmosphere at 5% CO₂ and fresh medium was given every other day. C17.2 cells were passaged (1/10) when reaching 90% confluency. PC12 cells were grown in 25 cm² cell culture flasks (Corning, Amsterdam, The Netherlands) which were coated with collagen (rat tail collagen type I, Invitrogen, Belgium) and passaged (1/5) when growing in small clumps (approximately 5 cells/clump and reaching 70-80% confluency). Fresh medium was given every other day. Primary human umbilical vein endothelial cells (HUVECs) were kindly provided by Dr. Aldo Ferrari (ETH Zurich, Switzerland). For cultivation, cells were kept in 75 cm² cell culture flasks (Corning, Amsterdam, The Netherlands) which were coated with collagen (rat tail collagen type I, Invitrogen, Belgium) prior to cell seeding. The cells were maintained in endothelial cell basal growth medium and growth supplement (Cell Applications, Tebu-Bio, Le Perray en Yvelines, France) and passaged (1/5) when reaching 80-90% confluency. Every other day, fresh medium was given.

II.2) Cellular NP uptake studies.

All cell-NP interactions studies were performed according to standard protocols as described elsewhere^[7]. For cellular uptake studies, C17.2, HUVEC or PC12 cells were seeded in 25 cm² cell culture flasks coated with rat tail collagen type I and allowed to settle overnight. Next, the cells were incubated with the Au NPs at 50 nM for 24 h at 37°C and 5% CO₂. Then the medium was removed, cells washed three times with phosphate buffered saline (PBS) after which the cells were lifted by trypsin (0.025%; Gibco, Invitrogen, Belgium), and pelleted by centrifugation. The cells were then fixed for 20 min in 2% paraformaldehyde (PFA) after which they were washed three times with PBS. Cells were then pelleted at 4·10⁵ cells/condition in 0.2 M cacodylate buffer (pH 7.4), and postfixed for 1 h at 4 °C with 1% osmium tetroxide in the same buffer. Cell pellets were dehydrated with ethanol, then embedded in Epon-Araldite. Thin (70 nm) sections were stained successively with 5% uranyl acetate and 1% lead citrate. TEM observation was performed with a FEI CM120 operated at 120 kV, equipped with 2k x 2k USC-1000 slow-scan CCD cameras (Gatan, CA) at a nominal magnification of x 45,000.

For quantitative cellular uptake levels, a similar setup was used, where C17.2, HUVEC or PC12 cells were seeded in 25 cm² cell culture flasks coated with rat tail collagen I and allowed to settle overnight followed by incubation with the PEGylated Au NPs at 0, 50, 100, 200, 400 or 800 nM for 24 h after which the medium was aspirated, cells washed 3 times with PBS, lifted by trypsin and pelleted by centrifugation prior to fixation in PFA. Following fixation, cells were washed three times with PBS and kept as a pellet of 4·10⁵ cells/condition in 0.5 mL PBS to which 2 mL of aqua regia was added. The samples were then microwave digested (MLS 1200 Mega, Milestone, Shelton, CT, USA) and appropriately diluted in ultrapure water (> 18.2 MΩ.cm resistivity). Iridium (Merck, Darmstadt, Germany) was added as an internal standard (final concentration: 2 µg.L⁻¹). External calibration was applied to quantify the amount of gold. External standard were obtained by diluting a standard gold

solution (ALFA Johnson Matthey, Karlsruhe, Germany) in the same background solution as the samples. All samples were measured in triplicate using the following settings: rf power: 1,150 W, plasma gas flow rate: 15 L/min, auxiliary gas flow rate: 0.85 L/min, nebulizer gas flow rate: 1.15 L/min. For sample introduction a MicroMist nebulizer (200 µL/min) and a cyclonic spray chamber both from Glass Expansion (Pocasset, MA, USA) were used. To determine the number of Au NPs per cell, pellets of a known number of cells (in the range of $4 \cdot 10^5$ cells/condition) were used for inductively coupled plasma mass spectrometry (ICP-MS) analysis. Making dilutions of the stock solution of the Au NPs of known concentration (2.62 µM Au NPs), the weight (= number of gold atoms per NP: ≈ 3900) could be determined. This experimental result can be compared with the number of gold atoms per NP calculated from the TEM data. Then, considering an average particle diameter of $d_c = 4.6$ nm and taking into account the spherical shape of the NPs, the volume of one NP is:

$$V_{NP} = 4/3 \cdot \pi \cdot (d_c/2)^3 = 50.965 \text{ nm}^3$$

The atomic gold density is $\rho = 19.30 \text{ g/cm}^3$ and thus the mass m_{NP} of one Au NP (only the inorganic core is considered) as:

$$m_{NP} = \rho \cdot V_{NP} = 19.30 \text{ g/cm}^3 \cdot 50.965 \text{ nm}^3 \cdot 10^{21} \text{ cm}^3/\text{nm}^3 = 9.8 \cdot 10^{-19} \text{ g}$$

The number of gold atoms per Au NP is estimated as:

$$N_{Au/NP} = N_A \cdot m_{NP} / M_{Au} = 6.022 \cdot 10^{23} \text{ mol}^{-1} \cdot 9.8 \cdot 10^{-19} \text{ g} / 196.97 \text{ g/mol} \approx 3000$$

Hereby N_A is the Avogadro constant and M_{Au} is the molecular weight of gold. This result is close to the experimental data as obtained by ICP-MS of $N_{Au/NP} = 3900$.

Please note that the assessment of NP concentration is quite critical for this analysis and that the accuracy of the results obtained is therefore dependent on the accuracy of the NP concentration determination which was done as described above. Therefore, the difference in values obtained (3000 vs 3900 Au atoms per NP) likely stems from slight inaccuracies in determining the stock concentration of Au NPs as well as the assumptions made for the theoretical calculations, being perfectly spherical particles of exactly 4.6 nm diameter. As an example, after ICP-MS measurement of the cell samples, the concentration of gold (weight: *e.g.* 0.48 µg/L Au for HUVEC cells treated with 100 nM Au NPs, taking into account the dilution steps) in every sample could be determined. As the molecular weight of Au equals $M_{Au} = 197 \text{ g/mol}$, this value can be converted to the number of Au atoms per sample ($0.48 \text{ µg/L} / 197 \text{ g/mol} \cdot 6.022 \cdot 10^{23} \text{ mol}^{-1} \approx 1.46 \cdot 10^{15} \text{ L}^{-1}$) which equals $3.74 \cdot 10^{11} \text{ L}^{-1}$ Au NPs (by dividing by $N_{Au/NP} = 3900$). As the number of cells in this condition equaled $\approx 407,000 \text{ cells/L}$ this leads to $9.2 \cdot 10^5 \text{ Au NPs/cell}$. The cellular uptake efficiency could then be calculated by converting the concentration of NPs in the cell medium to the total number of NPs present (by multiplying with Avogadro's number and taking the total volume into account). Next, the number of NPs/cell can be multiplied by the exact number of cells used for the analysis to get the total number of cell-associated NPs. By dividing the number of cell-associated NPs by the total number of NPs used for cell labeling, the cellular uptake efficiency can be calculated, as shown in Supporting Table S3.

c [nM]	PC12	HUVEC	C17.2
50	2.4	5.2	2.9
100	3.9	9.2	4.5
200	6.9	16.7	7.8
400	12.2	27.4	13.3
800	19.6	46.4	22.9

Supporting Table S2: Cellular levels of PEGylated Au NPs (in 10^5 NPs/cell) as detected via ICP-MS upon different incubation concentrations c [nM] of Au NPs.

c [nM]	PC12	HUVEC	C17.2
50	0.26% (x7.5)	0.57% (x4.0)	0.32% (x8.6)
100	0.21% (x7.9)	0.51% (x4.1)	0.25% (x10.0)
200	0.19% (x7.7)	0.46% (x3.7)	0.22% (x 9.5)
400	0.17%	0.38%	0.18%
800	0.14%	0.32%	0.16%

Supporting Table S3: Cellular uptake efficiencies of PEGylated Au NPs determined by dividing the total number of cell-associated NPs over the total number of NPs originally provided in the incubation medium. The values between parentheses indicate how much the uptake value for PEGylated Au NPs is lower than for non-PEGylated Au NPs at identical concentrations. The data for the non-PEGylated Au NPs were taken from Soenen et al.^[7c].

II.3) Assessment of cell viability.

Quantitative cell viability data were generated using an dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Bornem, Belgium) assay (Promega, Madison, USA). For this, C17.2, HUVEC or PC12 cells were seeded at $5 \cdot 10^4$ cells/well in 96-well plates (200 μ L/well total volume) and allowed to settle overnight. Next, cells were incubated with the PEGylated Au NPs at 0, 10, 20, 50, 100, 200, 400 or 800 nM for 24 h, after which the MTT assay was performed according to the manufacturer's protocol (25 μ L of a 5 mg/ml solution of MTT salt added to every well and incubated for 4 h). Untreated cells were assayed as a control reference and untreated cells exposed to 0.1% Triton X-100 for 15 min were used as negative controls.

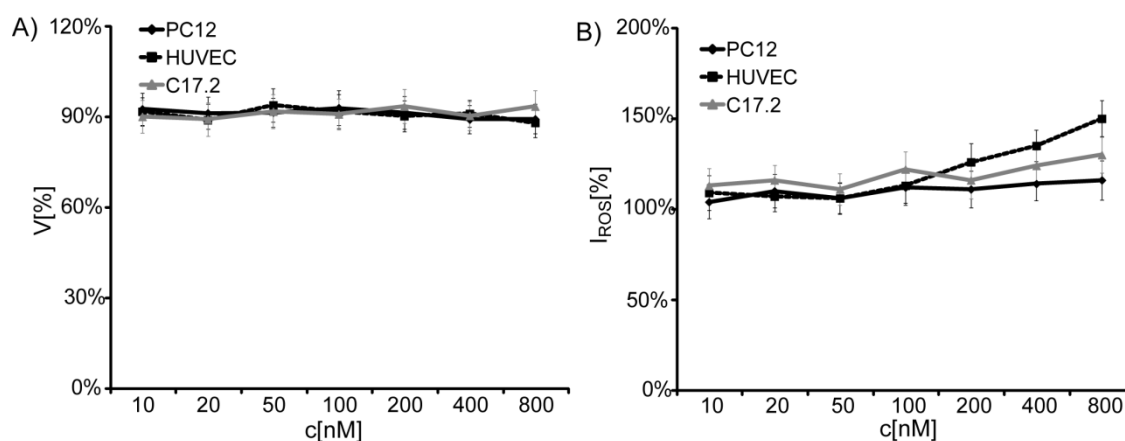
To assess the effect of N-Acetyl-L-cysteine (NAC; Sigma-Aldrich, Bornem, Belgium) on cell viability, C17.2, HUVEC or PC12 cells were seeded at $5 \cdot 10^4$ cells/well in 96-well plates (200 μ L/well total volume) and allowed to settle overnight. The cells were then pre-incubated with fresh medium containing 5 mM of NAC for 2 h followed by incubation with medium containing the PEGylated Au NPs at 0, 10, 20, 50, 100, 200, 400 or 800 nM for 24 h in the presence of 5 mM NAC. Then an MTT assay was performed as described above. Measurements were performed using a Wallac Envision platereader instrument (Perkin Elmer, Zaventem, Belgium) where absorbance was measured at 570 nm (MTT signal) and 630 nm (background signal). These experiments were repeated 5 times.

II.4) Controls included to evaluate NP-assay interactions.

Additional controls were included to assess whether the Au NPs interfered with the MTT assay readout: 1) Au NP-incubated cells were also treated with 0.1% Triton X-100 for 15 min and were found to lead to similar toxicity levels as the negative controls, indicating that the presence of Au NPs did not obscure the detection of cell death. 2) Also, medium which contained the Au NPs without any cells was analyzed in the same way as treated cells, showing no signal for MTT higher than the background signal which was subtracted, indicating that the NPs do not interfere with the assay readout itself at the concentrations used in the present study. 3) As a last control, cells were incubated with the Au NPs and an MTT assay was performed similar to the normal protocol but with omission of the tetrazolium salt, which resulted in no signal obtained. Again this data confirmed that the NPs did not interfere with the assay readout at the concentrations used in the present study.

II.5) Assessment of ROS.

To measure induced ROS levels, C17.2, HUVEC or PC12 cells were seeded at 5×10^4 cells/well in non-transparent 96 well plates (Greiner Bio One, Wemmel, Belgium) and allowed to settle overnight after which the cells were incubated with the PEGylated Au NPs at 0, 10, 20, 50, 100, 200, 400 or 800 nM for 24 h. Then, the cells were washed 3 times with PBS and incubated with 10 μ M 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA; Molecular Probes, Invitrogen, Merelbeke, Belgium) for 45 min followed by an additional incubation for 30 min in full culture medium. Then, the cells were washed twice with PBS and the fluorescence signal was measured using a Wallac Envision plate reader instrument with an excitation filter of 480 nm and an emission filter of 540 nm. For a positive control, cells were incubated with 1% H₂O₂ for 2 h prior to incubation with CM-H₂DCFDA. Data are expressed relative to untreated control cells (= 100%) as mean \pm SEM ($n = 5$). Additional controls treated similarly but without addition of CM-H₂DCFDA or the addition of Au NPs and CM-H₂DCFDA in the absence of cells did not give any significant signals, verifying the induction of ROS in the cells due to the Au NPs and lack of Au NP interference with the ROS assay. To assess the effect of NAC, a similar experiments was carried out where cells were pre-incubated with 5 mM NAC for 2 h after which they were incubated with the Au NPs in the presence of 5 mM NAC for 24 h and ROS levels were measured as described above.



Supporting Figure S9: Effects of PEGylated Au NPs in dependence of their incubation concentration c [nM] on A) cell viability V [%] and B) relative ROS induction I_{ROS} [%] when

cells were co-incubated with 5 mM NAC, a free radical scavenger. Data are expressed relative to untreated control cells as mean \pm SEM ($n = 5$). When appropriate, the degree of significance is indicated (: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).*

II.6) Assessment of DNA damage.

The occurrence of DNA double strand breaks was determined by staining HUVEC cells for phosphorylated γ -H₂Ax. Cells were seeded in collagen-coated non-transparent 96-well plates at 1×10^4 cells/dish in 200 μ L/well of full culture medium. Cells were allowed to settle overnight prior to being incubated with the Au NPs for 24 h at 0, 100, 200, 400 or 800 nM. Next, medium was aspirated, cells washed three times with PBS and fresh medium without Au NPs was given and cells were kept in culture for an additional 24 h. Then cells were washed three times with PBS, fixed in 2% PFA for 20 min at ambient temperature, permeabilized with 1% Triton X-100 for 15 min at ambient temperature and blocked with 10% goat serum-containing PBS for 30 min. The cells were then incubated with primary rabbit anti-phosphorylated γ -H₂Ax antibody (1 μ g/mL; Abcam, Cambridge, UK) for 2 h followed by 1 h incubation with Alexa Fluor 546-conjugated goat anti-rabbit antibody (1/250; Molecular Probes, Invitrogen, Merelbeke, Belgium) at ambient temperature. To get quantitative data, the fluorescence intensity levels were measured using a Wallac Envision platereader instrument with excitation at 550 nm and emission at 580 nm. These assays were repeated a total of 4 times.

II.7) Determination of mitochondrial membrane potential.

For determination of mitochondrial membrane potential ($\Delta\Psi_m$), HUVEC cells were seeded at 2×10^4 cells/well in non-transparent 96 well plates (Greiner Bio One, Wemmel, Belgium) and allowed to settle overnight. Cells were then incubated with the PEGylated Au NPs at 0, 100, 200, 400 or 800 nM for 24 h after which the medium was removed and cells were incubated for 30 min with 20 μ M of JC-10 (Enzo Life Sciences, Zandhoven, Belgium) at 37°C. For quantitative analysis, the cells were washed 3 times with PBS and the plate was then measured using a Wallac Envision platereader with an excitation filter of 480 nm and emission filters of 520 and 590 nm. The data are expressed as the ratio of green (damaged mitochondria) over red (healthy mitochondria) fluorescence as mean \pm SEM ($n = 4$).

II.8) Determination of cytoplasmic calcium levels.

For determination of cytoplasmic calcium levels, HUVEC cells were seeded at 2×10^4 cells/well in non-transparent 96 well plates (Greiner Bio One, Wemmel, Belgium) and allowed to settle overnight. Cells were then incubated with the PEGylated Au NPs at 0, 100, 200, 400 or 800 nM for 24 h after which the medium was removed and cells were incubated for 45 min with 5 μ M of Fluo-4 AM (Molecular Probes, Merelbeke, Belgium) at 37°C, followed by 30 min post-incubation in full medium prior to measuring the fluorescence intensity levels using a Wallac Envision platereader with an excitation filter of 490 nm and emission filters of 520 nm. These assays were repeated 4 times.

II.9) Determination of cell morphology.

For analysis of cytoskeletal deformations, HUVEC cells were seeded in collagen-coated 35 mm diameter glass bottom MatTek dishes (MatTek Corporation, Ashland, MA, USA) at 2×10^4 cells/dish in 1.5 mL of full culture medium. Cells were allowed to settle overnight prior to being incubated with the PEGylated Au NPs for 24 h at 0, 100, 200 or 400 nM. Next, medium was removed, cells were washed twice with PBS and fresh medium without any NPs was given and cells were kept in culture for an additional 24 h. Then cells were washed three times with PBS, fixed for 20 min in 2% PFA, permeabilized with 1% Triton X-100 for 15 min and blocked using 10% goat serum (Gibco, Invitrogen, Belgium)-containing PBS for 30 min. Then, cells were incubated with primary murine antibody in blocking buffer either against Vinculin (1 μ g/mL; Abcam, Cambridge, UK) for staining of focal adhesions or with primary antibody against α -tubulin (1 μ g/mL; Abcam, Cambridge, UK) for 2 h at ambient temperature. Cells were washed three times with blocking buffer after which they were incubated with secondary AF488-conjugated goat anti-murine IgG antibody (1/250 dilution; Molecular Probes, Invitrogen, Belgium) and AF546-conjugated phalloidin (1/300 dilution; Molecular Probes, Invitrogen, Belgium) for 1 h at ambient temperature. Cells were then washed three times with PBS and maintained in 1.5 mL PBS at 4°C prior to viewing by a Nikon Cs1 confocal laser scanning microscope (CLSM, Nikon Belux, Brussels, Belgium). For calculations of cell areas, fluorescence images were taken at a 20x magnification and areas were calculated using ImageJ software (NIH, USA) for at least 150 cells/sample and images were collected from at least 3 differently prepared samples.

II.10) Determination of pY397-FAK levels

C17.2 or HUVEC cells were seeded in 25 cm² tissue culture falcons at 1×10^5 cells/dish and allowed to settle overnight after which the cells were incubated with the PEGylated Au NPs at 0, 100, 200, 400 or 800 nM for 24 h. Then, media were removed, cells washed three times with PBS, and kept in culture in fresh culture medium for 43 h. Next, half the medium was then removed, fresh complete medium was added with or without sodium orthovanadate (200 μ M, three samples per concentration in the presence and absence of sodium orthovanadate; Sigma-Aldrich, St. Louis, MO, USA), and the mixture was incubated for another 5 h at 37°C, 5% CO₂. Next, the medium was aspirated and the cells were washed with icecold PBS, lifted by scraping, centrifuged at 300 g, and washed twice with PBS. Proteins were then extracted by lysing the cell pellets in 0.5 mL cell extraction buffer [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 10mM, pH 7.4), NaCl (100 mM), EDTA (2 mM), NaF (1 mM), Na₃VO₄ (5 mM), Triton X-100 (1%), glycerol (10%), sodium dodecyl sulfate (0.1%), phenylmethylsulfonyl fluoride (1 mM, Sigma-Aldrich, St. Louis, MO, USA), and protease cocktail inhibitor (cOmplete Mini, Roche Diagnostics GmbH, Mannheim, Germany)] for 30 min on ice with vortexing every 10 min. The extract was transferred to microcentrifuge tubes and centrifuged at 12,000 rpm for 10 min at 4°C. For determination of pY397-FAK levels, 10 μ L of the sample was diluted to 50 μ L with standard diluent buffer provided with the FAK (pY397) ELISA kit (cat. # KHO0441; Invitrogen, Camarillo, CA). The rest of the assays were carried out according to the respective protocols as provided by the manufacturer. For all the different protein extracts, 25 μ L aliquots were taken and the amount of protein was determined by means of a bicinchoninic acid (BCA) assay (Pierce, Rockford, USA) to deduce the number of cells per sample. The FAK and pY397-FAK levels were then normalized per 5×10^4 cells and expressed as the amount relative to the total FAK

present in untreated control cells (100%). This assay was repeated three times to allow statistical analysis.

II.11) Assessment of NFκB pathway activation.

C17.2, HUVEC and PC12 cells were seeded in 25 cm² collagen-coated tissue culture flasks at 1×10^5 cells/flask and allowed to settle overnight. Next, cells were incubated with fresh media (5 mL) containing the PEGylated Au NPs at 0, 100, 200, 400 or 800 nM for 24 h. Media were removed, cells washed twice with PBS, lifted with trypsin and pelleted by centrifugation at 0.3 g. Then, the pellets were resuspended in 1x Lysis buffer Mix (total volume 100 μL) according to the manufacturer's instructions (InstantOne ELISA NFκB pathway, eBioscience, Vienna, Austria) for 10 min at room temperature. Then, for every cell type and Au NP concentration, 5 μL of the lysed pellet was transferred to a well of the InstantOne microplate strips and further diluted with 45 μL/well of 1x Lysis buffer Mix. The concentration of activated NFκB were then determined according to the manufacturer's instructions. For every cell pellet, 10 μL was also used for protein determination using the bicinchoninic acid (BCA) assay (Pierce, Thermo Scientific, Rockford, USA) according to the manufacturer's instructions. For every cell type, results obtained for samples from different Au NP concentrations were normalized against the value obtained untreated control cells at identical protein levels. Positive controls for activated NFκB were included in the kit and used as reference values. Values are expressed as relative to those obtained for untreated control cells (= 1) for a total number of three independent repeats.

II.12) Determination of PC12 neurite outgrowth.

To assess the ability of PC12 cells to produce neurites, PC12 cells were seeded in collagen-coated 35 mm diameter glass bottom MatTek dishes (MatTek Corporation, Ashland, MA, USA) at 2×10^4 cells/dish in 1.5 mL of full culture medium. Cells were allowed to settle overnight prior to being incubated with the PEGylated Au NPs for 24 h at 0, 50, 100, 200 or 400 nM. After incubation, cells were washed twice with PBS and fresh medium was given and cells were kept in culture for another 12 h. Then, cells were washed three times with PBS after which cells were given NGF induction medium, consisting of high glucose DMEM, supplemented with 1% fetal calf serum, 5% horse serum, 1 mM sodium pyruvate, 2 mM L-Glutamine, 1% penicillin/streptomycin and 100 ng/mL NGF (Sigma-Aldrich, Bornem, Belgium). Cells were kept in this medium for 48 h where after 24 h, half the medium was replaced by fresh induction medium. Samples were then fixed, permeabilized and stained for F-actin and α-tubulin and images were collected as described above. Using images acquired at a low magnification (20x) processing occurred using ImageJ, where the number and length of neurites - defined as having twice the length of the cell body - were calculated using an ImageJ plug-in called NeuronJ^[8]. Data are expressed as the average number of neurites of a certain length per cell as mean ± SEM. At least 150 cells were analyzed per sample to allow statistical analysis.

II.13) Statistical analysis.

All data are expressed as mean ± SEM unless indicated otherwise and analyzed using one-way analysis of variance (ANOVA). When comparing the different conditions to the same control group, the Dunnett *post-hoc* analysis method was used. In all cases, the degree of significance is indicated when appropriate (*p < 0.05; **p < 0.01; ***p < 0.001).

III) References

- [1] a) A. Riedinger, F. Zhang, F. Dommershausen, C. Rucker, S. Brandholt, G. U. Nienhaus, U. Koert, W. J. Parak, *Small* **2010**, *6*, 2590-2597; b) M. Brust, M. Walker, D. Bethell, D. J. Schiffrin, R. Whyman, *J Chem Soc Chem Comm* **1994**, 801-802.
- [2] A. Lendlein, B. F. Pierce, L. Ambrosio, D. Grijpma, *Acta biomaterialia* **2012**, *8*, 4199-4199.
- [3] a) C. A. J. Lin, R. A. Sperling, J. K. Li, T. Y. Yang, P. Y. Li, M. Zanella, W. H. Chang, W. G. J. Parak, *Small* **2008**, *4*, 334-341; b) T. Pellegrino, L. Manna, S. Kudara, T. Liedl, D. Koktysh, A. L. Rogach, S. Keller, J. Radler, G. Natile, W. J. Parak, *Nano letters* **2004**, *4*, 703-707; c) M. T. Fernandez-Arguelles, A. Yakovlev, R. A. Sperling, C. Luccardini, S. Gaillard, A. S. Medel, J. M. Mallet, J. C. Brochon, A. Feltz, M. Oheim, W. J. Parak, *Nano letters* **2007**, *7*, 2613-2617; d) F. Zhang, Z. Ali, F. Amin, A. Feltz, M. Oheim, W. J. Parak, *Chemphyschem* **2010**, *11*, 730-735; e) C. Geidel, S. Schmachtel, A. Riedinger, C. Pfeiffer, K. Mullen, M. Klapper, W. J. Parak, *Small* **2011**, *7*, 2929-2934.
- [4] M. Valledor, J. C. Campo, F. J. Ferrero, M. T. Fernandez-Arguelles, A. Sanz-Medel, *Ieee Imtc P* **2007**, 481-486.
- [5] T. Pellegrino, R. A. Sperling, A. P. Alivisatos, W. J. Parak, *J Biomed Biotechnol* **2007**.
- [6] R. A. Sperling, T. Pellegrino, J. K. Li, W. H. Chang, W. J. Parak, *Adv Funct Mater* **2006**, *16*, 943-948.
- [7] a) S. J. H. Soenen, M. De Cuyper, *Contrast Media Mol I* **2011**, *6*, 153-164; b) S. J. Soenen, J. Demeester, S. C. De Smedt, K. Braeckmans, *Biomaterials* **2012**, *33*, 4882-4888; c) S. J. Soenen, B. Manshian, J. M. Montenegro, F. Amin, B. Meermann, T. Thiron, M. Cornelissen, F. Vanhaecke, S. Doak, W. J. Parak, S. De Smedt, K. Braeckmans, *Acs Nano* **2012**, *6*, 5767-5783.
- [8] E. Meijering, M. Jacob, J. C. F. Sarria, P. Steiner, H. Hirling, M. Unser, *Cytom Part A* **2004**, *58A*, 167-176.